Elsevier Editorial System(tm) for Cellular Signalling

Manuscript Draft

Manuscript Number:

Title: INCREASED PLATELET ADHESION AND THROMBUS FORMATION IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

Article Type: Full Length Article

Keywords: Alzheimer's disease; platelets; amyloid; adhesion; thrombus formation

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Abstract: Vascular dysfunctions and Alzheimer's disease show significant similarities and overlaps. Cardiovascular risk factors (hypercholesterolemia, hypertension, obesity, atherosclerosis and diabetes) increase the risk of vascular dementia and Alzheimer's disease. Conversely, Alzheimer's patients have considerably increased predisposition of ischemic and hemorrhagic strokes. Platelets are major players in haemostasis and thrombosis and are involved in inflammation. We have investigated morphology and function of platelets in 3xTg-AD animals, a consolidate murine model for Alzheimer's disease. Platelets from aged 3xTg-AD mice are normal in number and glycoprotein expression, but adhere more avidly on matrices such as fibrillar collagen, von Willebrand factor, fibrinogen and amyloid peptides compared to platelets from age-matching wild type mice. 3xTg-AD platelets adherent to collagen also show increased phosphorylation of selected signaling proteins, including tyrosine kinase Pyk2, PI3 kinase substrate Akt, p38MAP kinase and myosin light chain kinase, and increased ability to form thrombi under shear. In contrast, aggregation and integrin □IIb□3 activation induced by several agonists in 3xTg-AD mice are similar to wild type platelets. These results demonstrated that Alzheimer's mutations result in a significant pre-activated state of circulating platelets, evident with the progression of the disease.

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Dr. Casoli has great experience on platelets and Alzheimer's disease

Opposed Reviewers:

Pavia, March 29th, 2016

Dear Editor,

I submit to you a manuscript entitled: "Increased platelet adhesion and thrombus formation in a mouse model of Alzheimer's disease" by Caterina Visconte, Barbara Oliviero, Gianni Guidetti, Marta Zarà, Giordano Pula, Mauro Torti and myself, to be considered for publication in Cellular Signalling.

This study investigated and characterised platelets isolated from a well consolidate mouse model of Alzheimer's disease, the triple 3xTg-AD. These mice contain three mutations associated with familial AD (APP Swedish, Tau MAPT P1301L and preselinin1 PSEN1 M146V) that result in overexpression of APP, overproduction of Aβ42 and hyperphosphorylation of Tau in neurons. We have demonstrated that aged-3xTg-AD platelets adhere more avidly on subendothelial matrices (fibrillar collagen, von Willebrand factor, fibrinogen and amyloid peptides) compared to platelets from age-matching wild type mice. Thrombus formation is also accelerated in 3xTg-AD.

This manuscript describes original studies, and contains data that have not been already published in any substantial part or submitted for publication elsewhere.
The manuscript has been read and approved by all the authors and there are no conflicts of interest.

The first two authors should be regarded as joint First Authors if possible (Canobbio Visconte)

I really hope that our work will meet the approval of the Editor and the Referees.

With Best Regards
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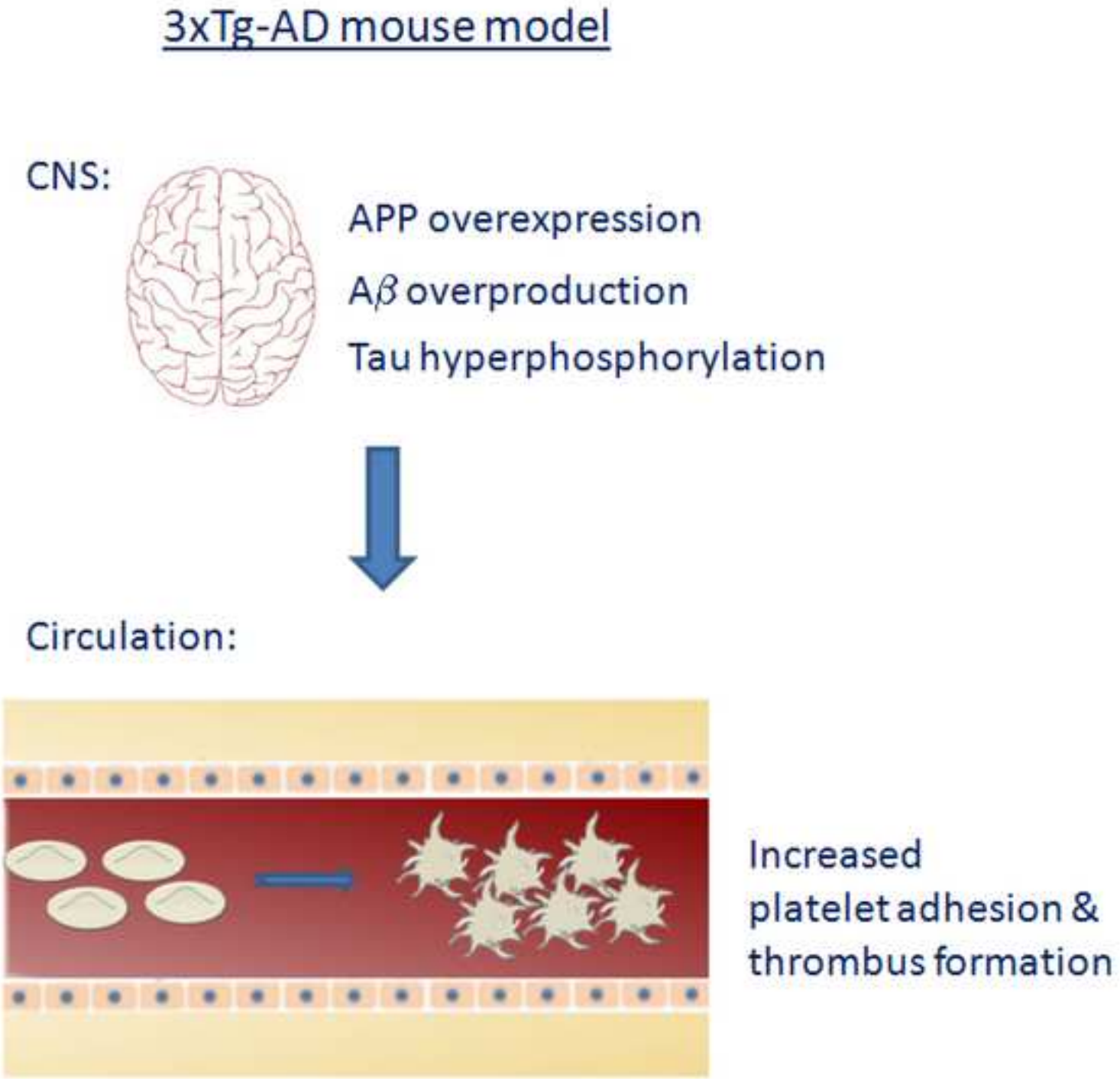
Graphical Abstract



HIGHLIGHTS

- Platelets are involved in vascular dysfunctions and Alzheimer's disease
- Platelet adhesion is increased in 3xTg-AD, a well consolidated mouse model of Alzheimer's disease
- Thrombus formation is also accelerated in 3xTg-AD
- Alzheimer's mutations result in a significant hyper-activated state of circulating platelets

ABSTRACT

Vascular dysfunctions and Alzheimer's disease show significant similarities and overlaps. Cardiovascular risk factors (hypercholesterolemia, hypertension, obesity, atherosclerosis and diabetes) increase the risk of vascular dementia and Alzheimer's disease. Conversely, Alzheimer's patients have considerably increased predisposition of ischemic and hemorrhagic strokes. Platelets are major players in haemostasis and thrombosis and are involved in inflammation. We have investigated morphology and function of platelets in 3xTg-AD animals, a consolidate murine model for Alzheimer's disease. Platelets from aged 3xTg-AD mice are normal in number and glycoprotein expression, but adhere more avidly on matrices such as fibrillar collagen, von Willebrand factor, fibrinogen and amyloid peptides compared to platelets from age-matching wild type mice. 3xTg-AD platelets adherent to collagen also show increased phosphorylation of selected signaling proteins, including tyrosine kinase Pyk2, PI3 kinase substrate Akt, p38MAP kinase and myosin light chain kinase, and increased ability to form thrombi under shear. In contrast, aggregation and integrin αIIbβ3 activation induced by several agonists in 3xTg-AD mice are similar to wild type platelets. These results demonstrated that Alzheimer's mutations result in a significant hyper-activated state of circulating platelets, evident with the progression of the disease.

1. INTRODUCTION

Alzheimer's disease (AD) is the most invalidating dementia in the elderly, affecting more than 45 million people worldwide with tendency to rise with the aging of the population [1]. AD is characterized by abnormal deposition of short amyloid β peptides ($A\beta_{140}$ and $A\beta_{1-42}$) in the brain parenchyma and in the cerebral vessels. The latter is often correlated with cerebral amyloid angiopathy [2]. Aβ peptides derive from the amyloidogenic metabolism of amyloid precursor protein APP by the actions of β and γ secretases. Alternatively APP may be proteolysed by α and γ secretases to release the non-toxic peptide p3 [3]. Another important feature of AD is the intracellular accumulation in neurons of the microtubule associated protein Tau, in its hyperphosphorylated form (neurofibrillar tangles) [4].

Although AD is prevalently known as a neurological disorder, it is now well accepted that it actually represents a systemic disease that affects peripheral tissues with alterations in blood cells and vessels. It has been demonstrated that AD is related to alterations of the vascular system and is associated to vascular disorders, including stroke, atherosclerosis and hypertension [5,6]. Conversely, many risk factors that predispose to vascular disorders may increase the risk for AD [7].

Among peripheral cells, platelets, which are responsible for haemostasis and thrombosis, are the most likely link between vascular disorders and AD [8-10]. Platelets express high amount of APP and the secretases that are responsible for its metabolism store APP soluble fragments and Aβ in α-granules and release Aβ in plasma upon platelet activation [11,12]. It is not clear whether Aβ present in plasma derives exclusively from platelets or comes from the brain through the blood brain barrier (BBB), but it has been demonstrated that Aβ is able to cross the BBB in pathological conditions [13,14]. Aβ peptides in plasma stimulate platelet activation [15-17], promote ROS production [18], platelet adhesion and thrombus formation [16,18]. Accumulation of Aβ in cerebral vessels also contributes to a chronic neuroinflammatory status that consecutively may exacerbate platelet activation and viceversa [19]. Platelets also express Tau and the presence of high molecular weight variants in AD patients has been suggested as a potential peripheral biomarker for AD [20,21].

Abnormalities of platelet morphology and APP metabolism have been described in platelets from AD patients [22], in particular APP ratio is decreased in platelets from AD patients compared to age-matched subjects [23,24]. Also, AD platelets show high level of activation in resting conditions, high P-selectin expression and integrin $\alpha_{IIb}\beta_3$ activation upon stimulation, high level of platelet/leukocytes aggregates and high number of pro-coagulant "coated platelets" [25-28]. Pre-activation of platelets has been observed also in a mouse model of AD, APP23 mice: platelets from aged APP23 mice show strongly enhanced integrin activation and degranulation. The hyperactivated state of platelets results in a pro-coagulant phenotype that increases and worsens vascular inflammation and thrombosis [29].

Here, we analyzed platelet activation in the triple 3xTg (3xTg-AD) mice. These mice contain three mutations associated with familial AD (APP Swedish, Tau MAPT P1301L and preselinin1 PSEN1 M146V) that result in overexpression of APP, overproduction of Aβ42 and hyperphosphorylation of Tau in neurons. Extracellular deposition of Aβ is evident in 6 months and Tau pathology in 12 months [30]. Platelets from 3xTg-AD mice show decreased platelet APP isoform, a situation that is commonly observed in AD platelets, and an increased level of soluble APPβ in plasma [31]. Moreover cerebrovasculature in 3xTg-AD mice shows markers of activation and overexpresses Aβ, thrombin, tumor necrosis factor α, interleukins 1 and 6 and MMP9 [19]. The activation state of platelets in 3xTg-AD mice has never been investigated. The current study explored platelet adhesion, activation and thrombus formation in >18 months 3xTg-AD mice compared to aged matched wild type control platelets.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

3xTg-AD mice were kindly provided by Dr. Dimtry Lim (University of Piemonte Orientale, Italy). 3xTg-AD mice were generated as described elsewhere [30]. The use of mice for our experimental work was approved by the Ethics Committee of the University of Pavia, according to the EU Directive 2010/63/EU for animal experiments. Thrombin, the thromboxane A2 analogue U46619, PGE1, apyrase, $A\beta_{25-35}$, bovine serum albumin, TRITC-conjugated phalloidin, fibrinogen and CFSE were purchased from Sigma-Aldrich. Bicinchoninic acid assay was from Thermo Fisher. Enhanced chemiluminescence (ECL) substrate was from Millipore. Convulxin was provided by Dr K. J. Clemetson (Theodore Kocher Institute, University of Berne, Switzerland). Collagen type I was kindly provided by Prof. M. E. Tira (University of Pavia, Italy). Von Willebrand factor (Koate) was from Bayer. Stromatol was from Mascia Brunelli. PE anti-Ly-6G/ly-6C(Gr-1), FITC anti-CD41 and PerCP anti-CD45 were from Biolegend. Anti-GPVI, anti-CD42b, anti-CD41 and anti-CD49b conjugated antibodies for flow cytometry were from Emfret Analytics. FITC-fibrinogen was from Molecular Probes. The monoclonal anti-tubulin (DM1A) was from Santa Cruz Biotechnology. Antibodies against APP: 22C11 were from Chemicon, and 6E10 from Covance. Anti-phospho Pyk2(Y402), anti-phospho Akt(S473), anti-phospho p38MAPK(T180-Y182) and anti-phospho MLC(S19) were from Cell Signaling Technology.

2.2 Platelet preparation

Murine platelets were prepared as previously described [10] from blood collected from the abdominal vena cava of anesthetized animals in syringes containing ACD/3.8% sodium citrate (2:1) as anticoagulant. Briefly, anticoagulated blood was diluted with HEPES buffer (10mM HEPES, 137mM NaCl, 2.9mM KCl, 12mM NaHCO3, pH 7.4) and centrifuged for 10 minutes at 180g to obtain platelet-rich-plasma (PRP). PRP was then transferred to new tubes and the remaining red blood cells were diluted with HEPES buffer and centrifuged again at 180g for 7 minutes. The upper phase was added to the previously collected PRP and 0.02 U/mL apyrase, and 1μM PGE1 were added before centrifugation at 550g for 10 minutes. The supernatant platelet-poor plasma was removed and the platelet pellet was washed in PIPES buffer (20mM PIPES, 136mM NaCl, pH 6.5) and centrifuged at 720g for 15 minutes. Platelet pellet was finally gently resuspended in 500μL of HEPES buffer, platelets were counted and the platelet count was adjusted to 5×10^8/ml. Upon addition of 5.5 mM glucose, cells were allowed to rest for 30 min at room temperature.

For aggregation studies, washed platelets from WT and 3xTg-AD mice (0.25 ml, 2×10^8 platelets/ml) were stimulated under constant stirring with 0.05U/ml and 0.1U/ml thrombin, 50ng/ml convulxin and 1μM U46619 in the presence of 1mM calcium in a Chronolog Aggregometer (Mascia Brunelli). Platelet aggregation was monitored continuously over 5 minutes.

2.3 Preparation and extraction of brain tissue

Brain was dissected from euthanized mouse and homogenized in 1 mL of ice-cold RIPA buffer (50mM Tris/HCl, pH 7.4, 200mM NaCl, 2.5mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 2mM PMSF, 100μg/ml leupeptin, 100μg/ml aprotinin, 2mM NaF, 2mM Na_3VO_4, pH 7.4) for 4 hours at 4°C. After homogenization, lysates were cleared at 18000g for 10 minutes at 4°C to eliminate debris and insoluble materials. Supernatants were collected and protein concentration was determined by bicinchoninic acid assay.

2.4 Flow cytometry

Flow cytometry experiments were performed essentially as described [32]. Samples of washed platelets (10^6 cells in 0.05 mL of HEPES buffer containing 0.1mM $CaCl_2$, 1mM $MgCl_2$, 5.5 mM Glucose and 0.1% BSA), untreated or stimulated with different doses of TRAP4, convulxin, U46619, ADP or $A\beta_{25-35}$ were labelled for 30 minutes at room temperature with FITC-conjugated fibrinogen. Surface expression of different glycoproteins on WT and 3xTg-AD mice were determined using specific antibodies: glycoprotein VI, CD42b, CD41 and CD49b. The reaction was stopped with PFA 0.5% and samples were analyzed by flow cytometry using a FACSCalibur instrument equipped with CellQuest Pro software (BD Biosciences). Data analyses were performed using the FlowJo Version 7.6.1 software (TreeStar).

2.5 Adhesion and spreading assay

Glass coverslips were coated overnight at room temperature with 100μg/ml fibrinogen, 25μg/ml collagen, 10μg/ml koate or 10μM $A\beta_{25-35}$, and then blocked with 1% BSA for 2h at room temperature. Washed platelets (0.5 ml; 4 $x10^7$ cells/ml) were added to dishes coated with fibrinogen and Aβ in the presence of 1mM $CaCl_2$, collagen in the presence of 2mM $MgCl_2$, and vWF in the presence of the cofactor botrocetin. Non adherent cells were discharged, and adherent platelets were fixed, permeabilized with Triton X-100, and actin filaments were stained by TRITC-conjugated phalloidin, as previously described [33]. Platelets were viewed on a fluorescence microscope (Olympus BX51), and digital images (400X) were acquired. The number of adherent cells was determined using the ImageJ Version 1.42 software.

2.6 Analysis of signaling in adherent platelets

Polystyrene dishes (60-mm) were coated overnight at room temperature with 25μg/ml fibrillar collagen. Dishes were washed three times with 2.5 mL of PBS, blocked with 2 mL 1% BSA for 2 hour at room temperature, and then washed again three times with PBS. Murine platelets (0.5 ml; 0.5 $x10^9$ cells/ml) were added to collagen-coated dishes in the presence of 2 mM $MgCl_2$ and 1 mg/mL BSA. After 60 min of incubation at room temperature, non-adherent cells were removed and dishes were washed three times with 2.5mL of PBS. For whole cell lysate preparation, adherent cells were directly solubilized by the addition

of 0.3 mL of 2% SDS in HEPES buffer, and then collected. Lysates were centrifuged at 18000g for 10 minutes and protein concentration was determined by bicinchoninic acid assay. Aliquots of each sample containing the same amount of proteins were used for immunoblotting analysis.

2.7 Electrophoresis and immunoblotting

Aliquots of platelet and brain lysates containing the same amount of proteins (15 μg and 150μg respectively) were separated by SDS–PAGE, and proteins were transferred to PVDF membrane. After blocking for 1 h with 5% BSA in TBS (20mM Tris/HCl, pH 7.5, 0.5mM NaCl), membrane was incubated overnight at 4°C with the desired primary antibody. In the present study the following antibodies and dilution were used: anti-APP (6E10), 1:1000; anti-APP(22C11), 1:1000; and anti-tubulin (DM1A), 1:2500; anti-phosphoPyk2 (Y402), 1:1000; anti-phosphop38MAPK(T180Y182) 1:1000; anti-phosphoMLC(S19), 1:1000; anti-phosphoAkt(Ser473)1:1000. Membrane was then extensively washed with 0.1%Tween20 in PBS and incubated with peroxidase-conjugated secondary antibody (1:2000) for 45 minutes. Upon extensively washing, reactive proteins were visualized with a chemiluminescence reaction, in a Chemidoc XRS (Biorad). Blots reported in the figures are representative images and the analysis of band intensity was performed by computer assisted densitometric scanning using Image J software.

2.8 Thrombus formation under flow

Thrombus formation was performed as previously described [34] with some modification. Briefly, glass coverslips were coated with fibrillar type I collagen (50μg/ml) and blocked with 1% BSA. Coverslips were mounted in a 50-μm-deep parallel-plate flow chamber (RC-31 from Warner instruments) under a fluorescence microscope, and rinsed with washing buffer (HEPES buffer supplemented with 2mM $CaCl_2$, 2mM $MgCl_2$, 5.5mM glucose, 0.1% BSA and 1U/ml of heparin). Blood was withdrawn by euthanized mice in PPACK/heparin, preincubated with 3 μg/ml CFSE for 15 minutes in the dark, and flowed over collagen at 1000/s for 5 minutes using a pump system (Harvard Apparatus PHD 2000). After perfusion the flow chamber was rinsed with washing buffer, and at least 10 fluorescence microscopic images were collected after 5 minutes of rinse. Images were analyzed by ImageJ Version 1.92 software and the extent of thrombus formation was calculated as the percentage of platelet covered area.

2.9 Data and statistical analysis

All of the reported figures are representative of at least 3 different experiments. Statistical analysis was performed using Prism Version 4 software(GraphPad) and data were compared by unpaired *t* test (media ± SEM).

3. RESULTS

3.1 Normal platelet count and glycoprotein expression in 3xTg-AD platelets

3xTg-AD mice represent a well-characterized AD model expressing mutations on human APP, Tau and presenilin1 associated with the familial AD. Transgenes are integrated at a single locus under the control of the neuronal Thy1.2 promoter, therefore their expression is restricted to the central nervous system. Since in this mouse model Aβ accumulates not only in brain but also in cerebral vessel walls [19] and in plasma [35], we verify the expression of amyloid precursor protein APP in brain tissue as well as in circulating platelets. We use two different antibodies: mAb 6E10 recognizes human APP and therefore is able to discriminate the transgenic human APP inserted under neuronal promoter; mAb 22C11 recognizes human and murine APP. As expected, immunoblotting analysis with mAb 6E10 reveals the presence of human APP in brain but not in platelet lysates of 3xTg-AD mice, confirming the specific neuronal expression of human APP in our transgenic model. Human platelets have been used as positive control: the slightly differences in the molecular weight of APP is due to different APP isoforms expressed in brain (mainly APP695) and platelets (mainly APP751/770). Immunoblotting with mAb 22C11 clearly shows that the level of expression of APP in brain of 3xTg-AD is two/three fold higher than in control WT mice, according to APP Swedish mutation [30]. On the contrary the level of expression of murine APP in platelets is normal and similar in WT and 3xTg-AD mice (figure 1A).

Although this mouse model have been widely use to investigate neuronal dysfunctions, little information is available on the consequences of these mutations on circulating blood cells, including platelets. Platelets express high level of APP [36]. We demonstrated that platelet and white blood cell number in 3xTg-AD mice are normal compared to WT (figure 1B and C). We next examined platelets for the surface expression of glycoprotein Ibα (CD42b), the immunoreceptor tyrosine-based activation motif (ITAM)–coupled collagen receptor GPVI, α2 (CD49b) and αIIb (CD41) integrins demonstrating no significant differences in glycoprotein expression between the two genotypes (figure 1D).

3.2 Activation and aggregation is normal in 3xTg-AD platelets

It has been previously demonstrated that platelets isolated from AD patients and AD transgenic mice (APP23) are in a pre-activated state and more prone to activation. Here, we analyzed the ability of 3xTg-AD platelets to be activated by different agonists in comparison to WT control platelets. We demonstrated that aggregation is not altered in 3xTg-AD platelets stimulated with the thrombin receptor activated peptide TRAP4, GPVI agonist convulxin, thromboxane analogue U46619 and Aβ peptides (figure 2A). Consistent with these results, integrin $\alpha_{IIb}\beta_3$ inside-out activation, measured as the ability of integrin to

bind fibrinogen, is similar in stimulated 3xTg-AD compared to WT platelets (figure 2B). We also studied α–granule secretion (analyzed as the % of platelets that expressed P-selectin on their surface) upon stimulation with different agonists, and demonstrated no significant differences between WT and 3xTg-AD mice (figure 2C).

3.3 Enhanced platelet adhesion and thrombus formation in 3xTg-AD mice

Platelet are able to adhere with different kinetics on physiological subendothelial matrices, collagen and von Willebrand factor, and fibrinogen. We have also demonstrated that platelets are able to specifically adhere on different amyloid peptides ($A\beta_{25-35}$, $A\beta_{1-40}$ and $A\beta_{1-42}$) in static conditions [37], a situation that is likely to occur in cerebral vessels upon deposition of amyloid peptides. Here we analyzed the ability of platelets from WT and 3xTg-AD mice to adhere to collagen, von Willebrand factor, fibrinogen and $A\beta$ in static conditions (adhesion time: 60 minutes). Adherent platelets were visualized in immunofluorescence after staining of actin filaments with phalloidin-TRITC. We observed that platelet adhesion is significantly increased in 3xTg-AD compared to control WT over all the substrates tested (figure 3A). The 3xTg-AD enhanced adhesion was also evident and significant at early time points (30 minutes) (supplementary data 1). In contrast, no differences in spreading were observed (data not shown). In order to evaluate whether adhesion results in active signalling in adherent platelets, we analyzed phosphorylation of selected signalling proteins in WT and 3xTg-AD platelets adherent to collagen. Phosphorylation of the focal adhesion kinase Pyk2, the PI3K substrates Akt, p38 MAPK, and myosin light chain (MLC) were analyzed using specific phospho-antibodies following 60 minutes of platelet adhesion to collagen. We observed increased phosphorylation of Pyk2 (Y402), Akt (S473), p38 MAPK (T180-Y182) and MLC (S18) in response to collagen adhesion in 3xTg-AD platelets compared to WT (figure 3B).

The enhanced ability of isolated 3xTg-AD platelets to adhere to collagen was also tested under flow conditions. Fluorescently labelled platelets in whole blood from WT and 3xTg-AD mice were perfused for 5 minutes at a shear rate of 1000/second over immobilized fibrillar collagen and thrombus formation was evaluated as percentage of area covered by adherent platelets. Figure 4 shows that platelet adhesion and thrombus formation on collagen-coated surface are significantly increased in 3xTg-AD compared to WT control platelets (figure 4A, B).

4. DISCUSSION

AD is a complex neuropathology that predominantly affects neurons and brain but also has a significant impact on peripheral tissues [38]. Accumulation of Aβ peptides in senile plaques in the brain is correlated with dementia and neuronal death. Aβ peptides also accumulate in cerebral vessel walls causing cerebral amyloid angiopathy [2]. In pathological conditions Aβ peptides may cross the BBB [13] and soluble or fibrillar Aβ peptides may activate circulating blood cells and endothelial cells [10]. We and others have demonstrated that Aβ peptides potentiate platelet activation induced by several agonists [15] and directly activate platelets, promoting release of intracellular granules content [16-18]. Platelet degranulation releases a small amount of platelet-derived Aβ peptides and inflammatory markers. Platelet-derived Aβ peptides in turn increase local Aβ concentration in plasma and activate neutrophils and endothelial cells, causing a chronic inflammatory state. It has been show that vascular activation is a relevant mechanism in AD pathogenesis [19]. Accordingly, AD patients show enhanced platelet activation and chronic inflammation that exacerbate peripheral tissue activation [25,27]. The chronic inflammation state has also been described in a murine model of AD, 3xTg-AD mice [19].

In this study we have analyzed morphology and function of platelets from aged 3xTg-AD mice (age > 18 month). We have demonstrated that 3xTg-AD platelets show enhance ability to adhere to components of the subendothelial matrix, such as collagen, von Willebrand factor and fibrinogen. Adhesion is the first step in platelet activation, and we have here demonstrated that 3xTg-AD platelets adherent to collagen activates intracellular signaling pathways. In particular, we observed that 3xTg-AD platelets adherent to collagen show a significant phosphorylation of selected signaling proteins, the tyrosine kinase Pyk2, the Akt kinase, which is activated downstream of PI3K, the mitogen activated kinase p38MAPK, and the myosin light chain kinase MLC, compared to WT adherent platelets. We have also demonstrated that 3xTg-AD platelets have an increase capacity to adhere over collagen under shear (1000/sec) and to form microthrombi. These results are in line with the observation by Jarre et al. that have demonstrated that APP23 transgenic mice that express human APP Swedish mutation (KM670/671NL) and overexpress APP in the nervous system show a prothrombotic phenotype *in vivo*, accompanied by shortened tail bleeding time and altered hemostasis [29]. Our results demonstrated that the hyperactivation state of circulating platelets is also detectable in the 3xTg-AD mice. However, in contrast to what observed in the APP23 mice, platelet from 3xTg-AD mice did not show any significant difference in aggregation, granule secretion and integrin activation in 3xTg-AD mice compared to WT. In contrast, major alterations of platelet function are related to their increased ability to adhere, become activated and form thrombi upon adhesion. Although these discrepancies may indicate that different mutations predisposing to AD may have peculiar consequence on platelet function, it cannot be excluded that they can be caused by different platelet preparation protocols affecting basal level of platelet activation.

Either WT and 3xTg-AD platelets do not express human APP, as expected, and we have shown in this study that the level of murine APP is similar in the two genotypes. Aβ peptides is increased in 3xTg-AD brain and cerebral vessels [30] and are also able to cross the BBB [13] suggesting that increased level of Aβ peptides in brain may result in increased Aβ plasma concentration. It has been recently demonstrated in 3xTg-AD mice that Aβ concentration in plasma during the first nine months of the pathology directly correlates with the progression of AD [35].

Our study has demonstrated that platelets from 3xTg-AD mice have an increased ability to adhere to immobilized Aβ peptides compare to platelets form control mice. Previously we have demonstrated that Aβ is a potent platelet agonist [16]. Therefore it is possible to hypothesize that in the onset of AD the progressive accumulation of Aβ in plasma may sensitize circulating platelets rendering them more prone to adhere to immobilized Aβ itself as well as to other adhesive proteins, a process that favours thrombus formation. This process may increase the cardiovascular risk associated to AD.

In conclusion our results indicate that blood platelets from 3xTg-AD mice are more susceptible to adhesion to subendothelial matrices and therefore may contribute to the vascular complications associated to the pathology.

Conflict of Interest

The authors declare no competing financial interests

Acknowledgments

We thank Dr. Dmitry Lim (University of Piemonte Orientale, Italy) and Dr. Francesco Moccia for support with the 3xTg-AD mice, and Professor Mario Umberto Mondelli (Fondazione IRCCS Policlinico San Matteo, Pavia, Italy) for flow cytometry facilities.

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FIGURE LEGENDS

Figure 1. Characterization of platelets from 3xTg-AD mice. (A) APP expression in brain and platelet lysates from WT and 3xTg-AD mice was investigated in immunoblotting by using two different antibodies against APP: 6E10 recognizes human APP and 22C11 recognizes human and murine APP. Human platelet sample was loaded as positive control. Tubulin was used for equal loading control. (B,C) Platelets and white blood cells (WBC) count in whole blood from WT (white bars) and 3xTg-AD (black bars) mice. Results are the means ± SEM of determination performed in 10 different mice. (C) Surface expression of different glycoproteins on WT (white bars) and 3xTg-AD (black bars) mice determined by flow cytometric analysis with specific antibodies. Data are expressed as the mean fluorescence intensity ± SEM of 4 different experiments performed in duplicate.

Figure 2. Aggregation, integrin $\alpha_{IIb}b_3$ inside-out activation and P-selectin expression of 3xTg-AD platelets. (A) Washed platelets (2×10^8/ml) from WT and 3xTg-AD mice were stimulated in an aggregometer with thrombin (0.05 and 0.1 U/ml), convulxin (50ng/ml), U46619 (1μM) and $A\beta_{25-35}$ (10 μM). Aggregation was monitored as the increase of light transmission up to 5 minutes. Traces in the figure are representative of at least 3 different experiments. (B) Flow cytometric analysis of FITC-labeled fibrinogen binding to WT (white bars) and 3xTg-AD (black bars) platelets stimulated with 0.5 mM TRAP4, 50ng/ml convulxin (CVX), 5 μM U46619, 10 μM ADP and 10 μM $A\beta_{25-35}$. Data are expressed as means ± SEM of 3 different experiments. (C) P-selectin (CD62P) expression on platelet surface is analyzed in flow cytometry (PE) in resting and stimulated conditions (thrombin 0.1 U/ml; convulxin 50ng/ml; U46619 5 μM + ADP 5 μM; $A\beta_{25-35}$ 10 μM) in either WT (white bars) and 3xTg-AD (black bars) platelets.

Figure 3. Adhesion and signaling of 3xTg-AD platelets. WT and 3xTg-AD platelets were let to adhere on glass coverlips coated with 25 μg/ml collagen, 10 μg/ml Koate (vWF), 100 μg/ml fibrinogen or 10 μM Aβ, as described in methods for 60 minutes. Adherent platelets were fixed, permeabilized and stained with TRITC conjugated phalloidin. Representative images at 400X magnification of adherent platelets to the indicated substrates are reported. Quantification of platelets adhesion, evaluated as number of adherent platelets/mm2, is reported on the right. WT (white bar), 3xTg-AD (black bars), *** $p<0.005$ (B) (i)Aliquots of whole lysate from platelets adherent to collagen were analyzed by immunoblotting with the following antibodies: P-Pyk2 (Y402), P-Akt(S473), P-p38MAPK (T180/Y182), P-MLC(S18). Tubulin was used as equal loading control. (ii) Quantitative evaluation of protein phosphorylation is reported in the histograms as the means ± SEM of 3 different experiments. WT (white bars) and 3xTg-AD (black bars). ***$P<$ 0.005.

Figure 4. Enhanced thrombus formation in 3xTg-AD. CSFE-labeled platelets in whole blood from wild-type (WT) and 3xTg-AD mice were perfused over immobilized fibrillar collagen at a shear rate of 1000/s for 5 minutes. Images were taken after brief rinse of the coverslips with washing buffer (2 minutes) and are reported in (A). Thrombus formation on the coverslips was evaluated by measuring the covered area in 10 different and randomly taken microscopic fields and results are reported in the histogram in (B) (WT, white bars; 3xTg-AD, black bars) as the means ± SEM of 3 different experiments.

Figure 1

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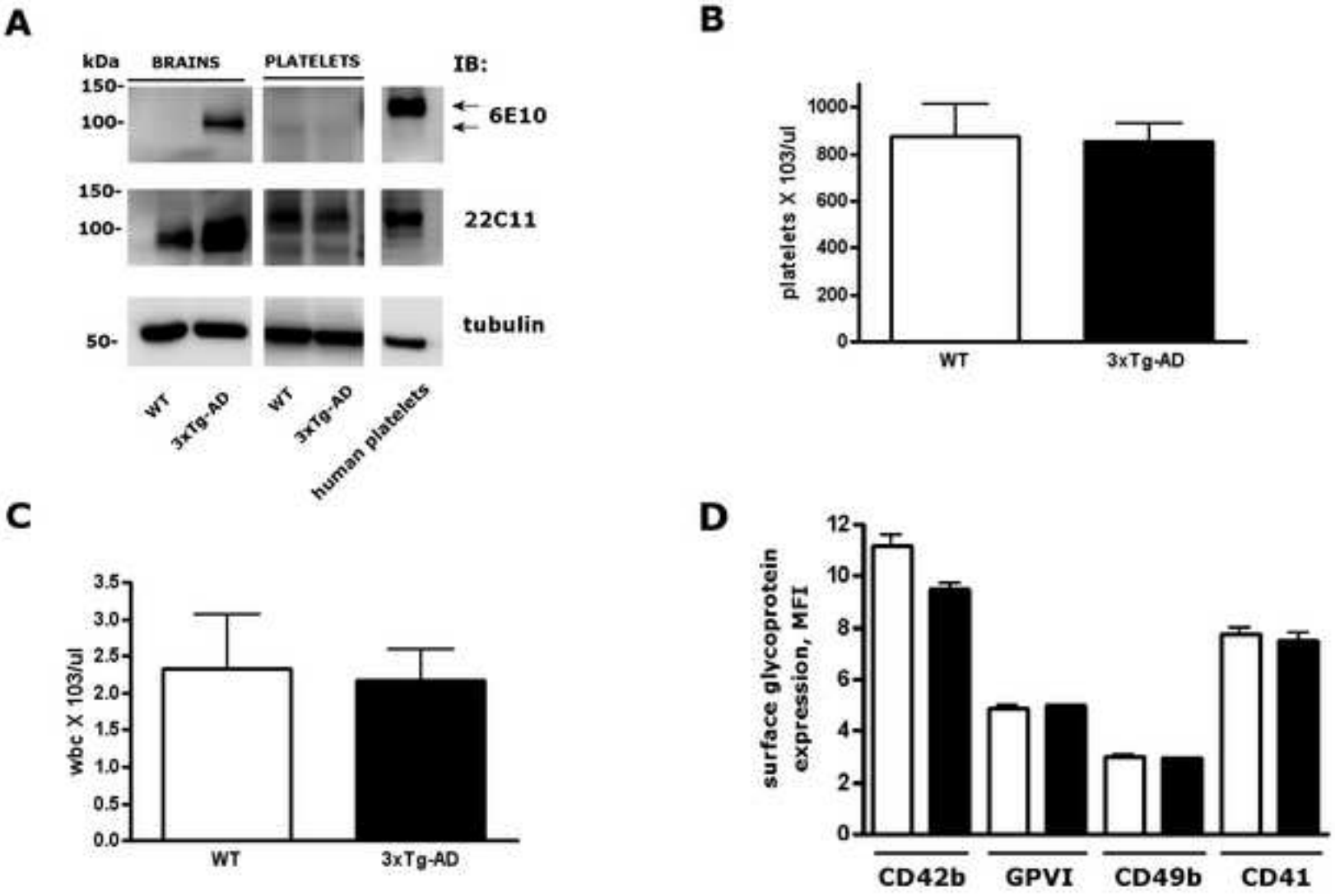

Figure 2

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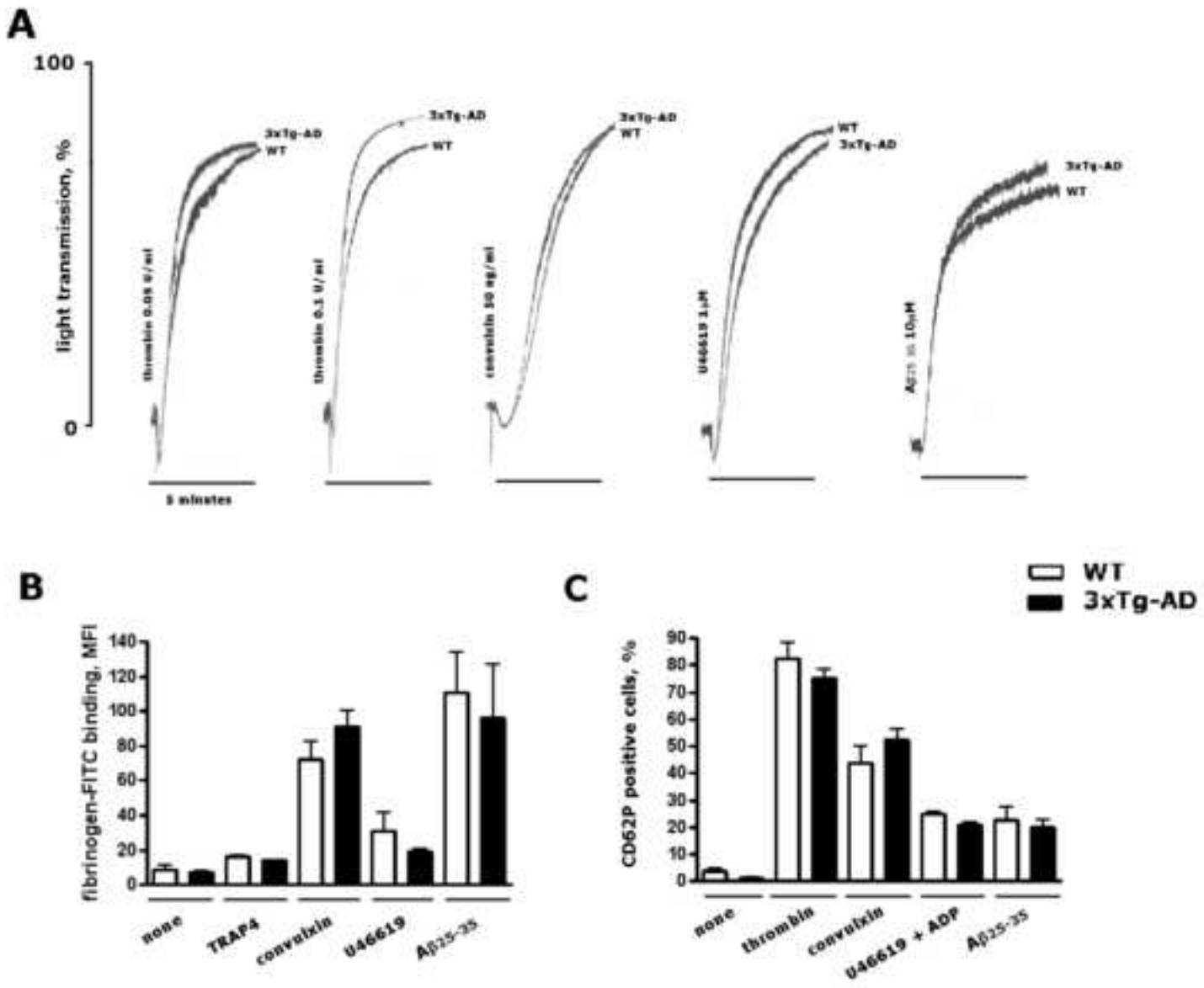

Figure 3A
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figure 3A

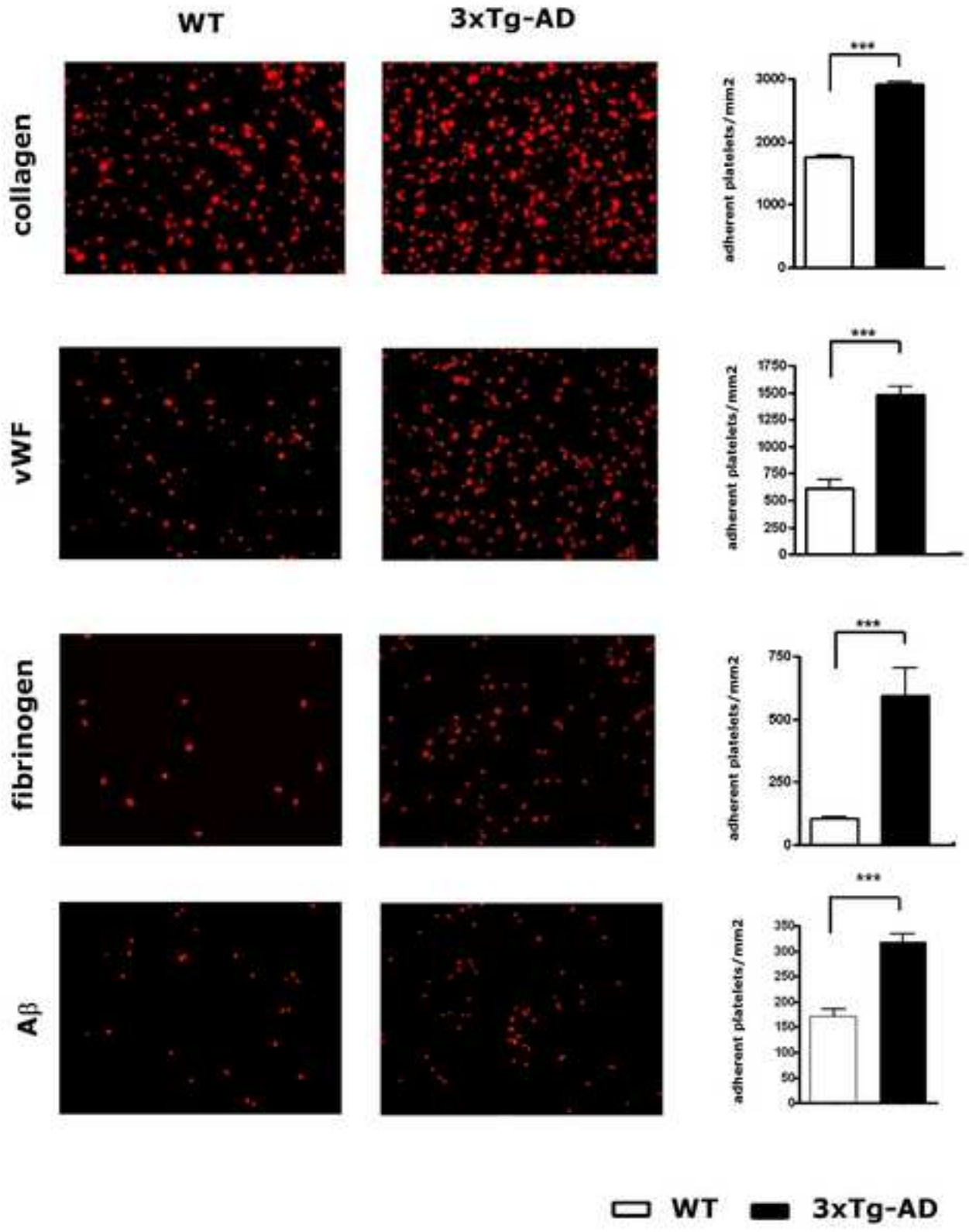

Figure 3B

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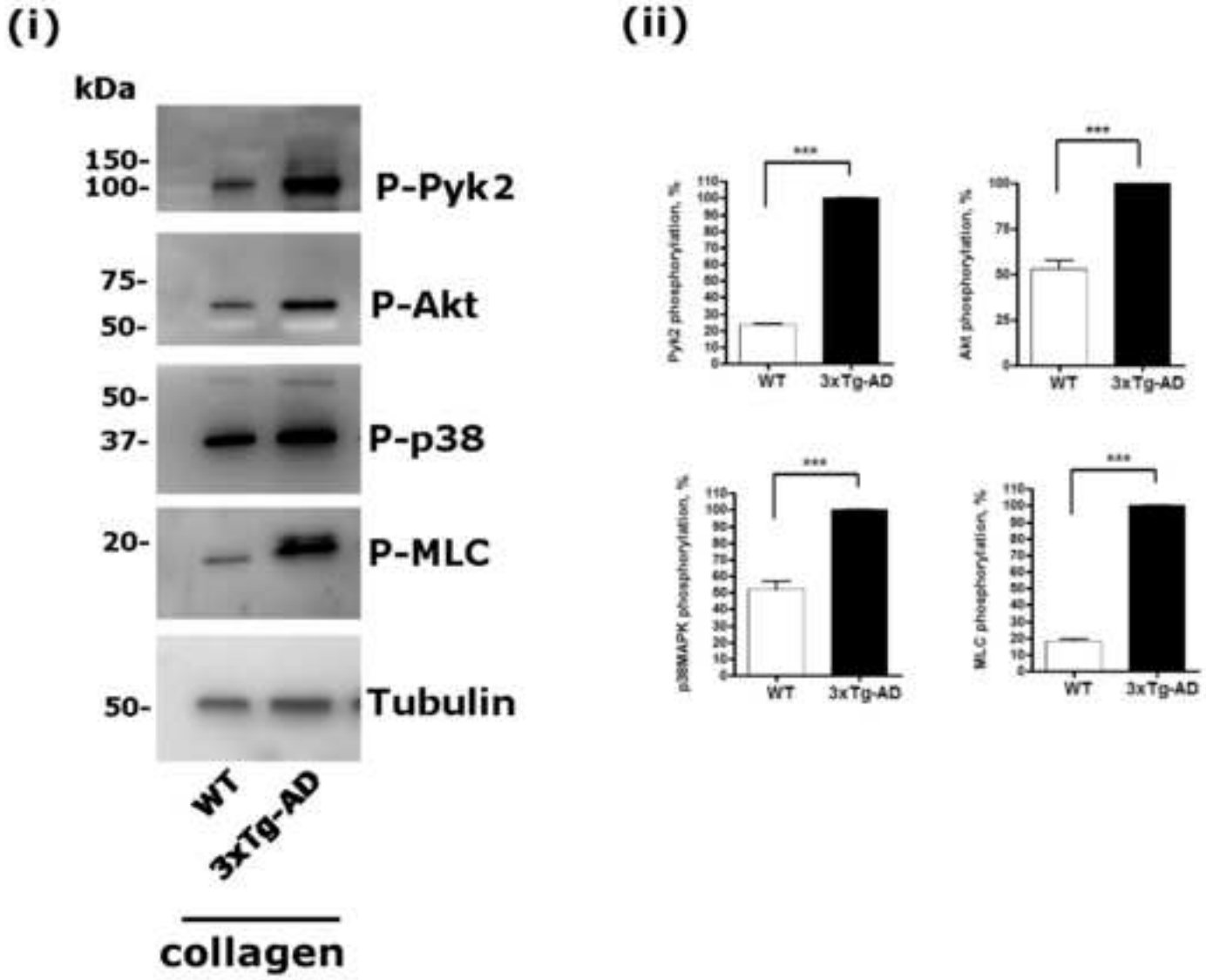

Figure 4
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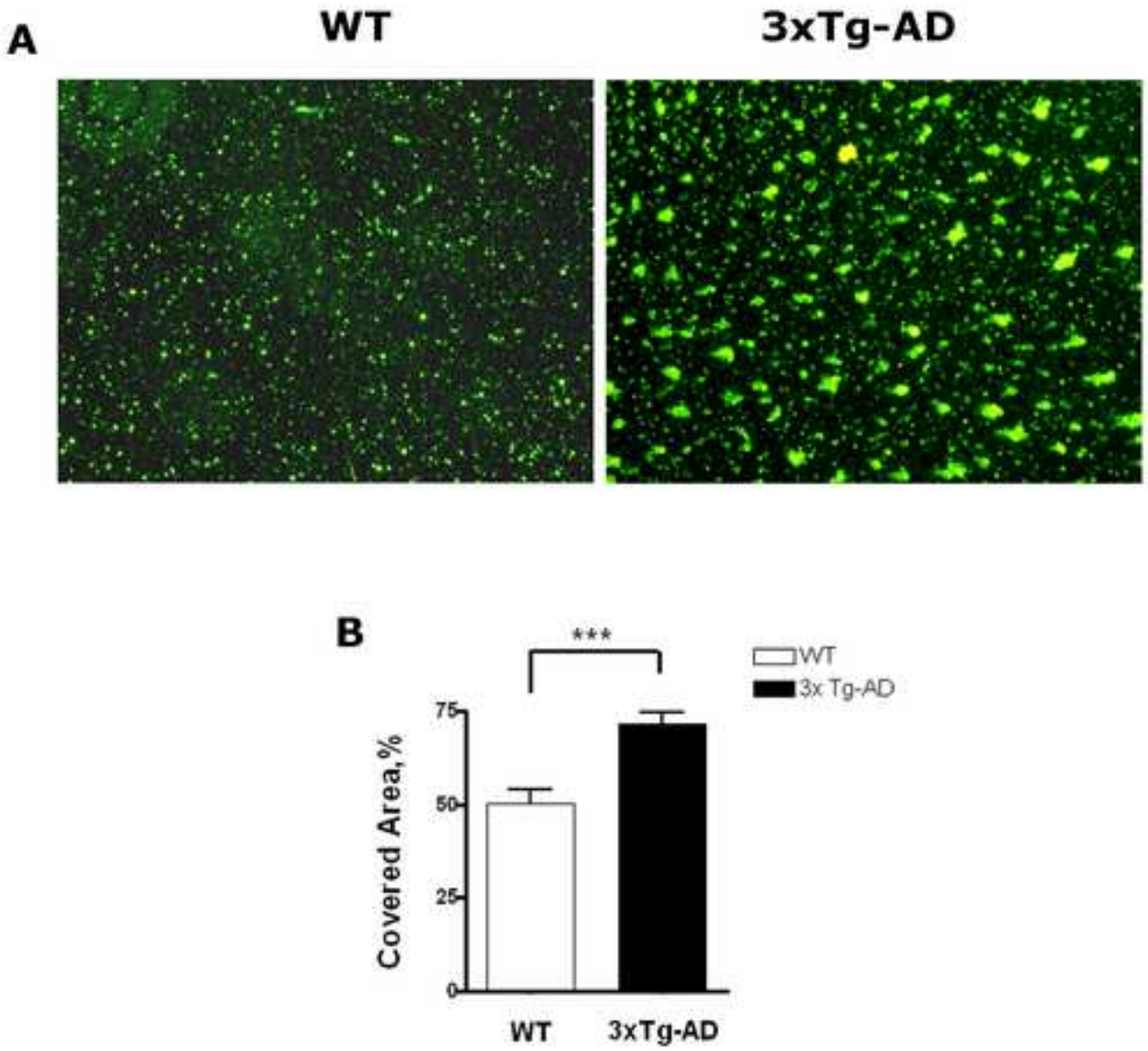

Supplementary Material

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